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Ozone Exposure and Pulmonary Metabolic Effects of Mediators and Hormones

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16. ABSTRACT

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FOREWORD

The many benefits of our modern, developing, industrial society are accompanied by certain hazards. Careful assessment of the relative risk of existing and new man-made environmental hazards is necessary for the establishment of sound regulatory policy. These regulations serve to enhance the quality of our environment in order to promote the public health and welfare and the productive capacity of our Nation's population.

The Health Effects Research Laboratory, Research Triangle Park, conducts a coordinated environmental health research program in toxicology, epidemiology, and clinical studies using human volunteer subjects. These studies address problems in air pollution, non-ionizing radiation, environmental carcinogenesis and the toxicology of pesticides as well as other chemical pollutants. The Laboratory participates in the development and revision of air quality criteria documents on pollutants for which national ambient air quality standards exist or are proposed, provides the data for registration of new pesticides or proposed suspension of those already in use, conducts research on hazardous and toxic materials, and is primarily responsible for providing the health basis for non-ionizing radiation standards. Direct support to the regulatory function of the Agency is provided in the form of expert testimony and preparation of affidavits as well as expert advice to the Administrator to assure the adequacy of health care and surveillance of persons having suffered imminent and substantial endangerment of their health.

The effort in these studies was largely directed at identifying the effects produced by acute ozone exposure and ascertaining the minimum dose of ozone required to produce each effect. These objectives were essentially achieved in the case of the increases of $PGF_{2\alpha}$ and TXB_2 in the lung. The minimal doses of ozone necessary to produce the two remaining observed effects remain to be determined.

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Director,
Health Effects Research Laboratory

ABSTRACT

Several physiological effects of ozone exposure involving smooth muscle contracting substances have been studied in Sprague-Dawley derived, male rats whose body weights were in the range of 180-250 grams and whose ages were in the range of 45-60 days. The ozone concentration of interest was 0.5-1.0ppm and the exposure time was four hours. However, higher concentrations of ozone and longer exposure times were sometimes employed. It was observed that the exposure of these rats to ozone concentrations of 0.5 or 1.0 ppm for 4.0 hours produced the following effects in their lungs: (a) caused the release of prostaglandin $F_2\alpha$ ($PGF_2\alpha$) and thromboxane A_2 (TXA_2), (b) increased the angiotensin converting enzyme activity, and (c) decreased the uptake of serotonin from the blood. Histamine and slow reacting substance of anaphylaxis (SRS-A) were not released nor was the histamine forming capacity of the lung altered. Edema formation was observed in the rats from one supplier, but not in those from another and succinoxidase activity was observed to be decreased by short exposure to ozone and increased by long exposure, as reported by previous investigators.

It was observed that the exposure of the rats to 0.5 ppm ozone for 4.0 hours prevented the release of $PGF_2\alpha$ and TXA_2 when the same animals were exposed 15 hours later to 2.0 ppm ozone for 4.0 hours. The significance of observation became apparent when it was found that animals which had been treated with the drug indomethacin were more susceptible to the lethal effects of ozone. This drug is widely recognized as a potent inhibitor of the enzyme cyclo-oxygenase (prostaglandin synthetase) which is required for the synthesis of all the prostaglandins including the thromboxanes and prostacyclines. It was therefore anticipated that the exposure of rats to a low dose of ozone and the administration of the drug indomethacin would affect the susceptibility of the animals to the lethal effects of ozone similarly. This result was observed experimentally and thus the smallest dose of ozone which can inhibit the release of $PGF_2\alpha$ and TXA_2 becomes of great concern as does the mechanism of this effect.

In contrast to the above, it was further observed that pretreatment of the rats with drugs which inhibit only the synthesis of TXA_2 resulted in decreased susceptibility of the animal to the lethal effects of ozone. This result is presumably due to the enhanced synthesis of $PGF_2\alpha$ in these circumstances. $PGF_2\alpha$, though less potent, is much more stable and presumably longer acting than TXA_2.

This report was submitted in fulfillment of Grant No. 804585 by Creighton University (Ibert C. Wells, project director) under the sponsorship of the U. S. Environmental Protection Agency. This report covers the grant period, October 20, 1976 to August 31, 1980.

GENERAL METHODS

Ozone exposure. Male rats, obtained from the Holtzman Company of Madison, WI and having body weights in the 180-250 g range (45-60 days of age), are placed in a closed, two compartment glass chamber through which air at room temperature (25°C) is passed at twelve liters per minute (0.42 CFM). The air is first passed through a silent electric discharge in order to convert some of the oxygen to ozone and is introduced into the chamber below the level of the rats and is exhausted from the chamber at the level of the rats' noses. Ozone concentration in the exhausted air is monitored by the spectrophotometric method of Saltzman [Anal. Chem. 31, 1914 (1959)] and is expressed as parts per million (ppm) as officially specified [Federal Register 36, (No. 84), 8196 (1971)]. In one instance, the rats were exposed to ozone at the Health Effects Research Laboratory, Environmental Protection Agency, Research Triangle Park, NC at which the ozone was generated by passing pure oxygen through ultraviolet irradiation. The concentration of ozone to which the animals were exposed was measured photometrically (EPA-600/4-79-057, Technical Assistance Document for the Calibration of Ambient Ozone Monitors).

Preparation of lung exudate. The intact lungs are removed from the decapitated rat, blotted to remove excess moisture, placed onto ice, and weighed. A small piece (50-100 mg) of lung is removed for the determination of water content by heating at 110°C to constant weight. The residual fresh lung tissue is reweighed and placed in a volume (ml) of cold Tyrode's solution equal to 2.5 times its weight (grams) and cut into about ten pieces with scissors. The mixture is allowed to stand on ice for 3-5 minutes and then the lung tissue is removed with forceps. The residual Tyrode's solution is the "lung exudate" and is used immediately or is stored at -20°C until used.

Preparation of lung extract. The pieces of lung tissue remaining from the preparation of the lung exudate are finely minced with a mechanical mincer (Mickle mincer, Brinkman Instruments) and transferred to a centrifuge tube and cold Tyrode's solution equal in volume (ml) to five times the original weight (grams) of the lung tissue is added. After this mixture is agitated for 30 seconds with the use of a vortex mixer, it is filtered through cheese cloth and the filtrate centrifuged at 1200 x g and at 2°C for 10 min. The supertant solution is the lung extract and is stored at -20°C until assayed. No differences were noted in the assay results from similar or identical samples stored overnight or for up to two weeks.

Assay of histamine and non-histamine contractile substances. A 3-4 cm segment from the distal end of the ileum of a normal guinea pig is suspended in a 10 ml bath of Tyrode's solution at 37°C using an isotonic muscle lever and transducer. The tissue and medium are gased with 95% O_2-5% CO_2. Solutions to be assayed are warmed to 37°C and 1.0 ml aliquots are added to the

bath and the contractions of the ileum are recorded by use of a physiograph. After each aliquot is tested, the tissue is washed 4-5 times with fresh Tyrode's solution at 37°C. The rapidly developing contractions produced by aliquots of lung exudates and extracts are compared with the contractions produced by known amounts of histamine. The results thus obtained are considered to measure the histamine contents of the exudates and extracts since they disappeared in the presence of antihistamine.

The non-histamine, smooth muscle contracting components in lung exudates and extracts are assayed in a similar manner except the Tyrode's solution used in the tissue bath contains 0.2 mg per liter each of atropine and of the antihistamine Pyrilamine (Merck). The contractions thus obtained when aliquots of lung exudates and extracts are added develop slowly and are compared with the previously obtained contractions produced by known amounts of histamine added to the same ileum preparation. This assay is considered to measure non-histamine, smooth muscle contracting substances such as prostaglandins, slow reacting substance of anaphylaxis (SRS-A), etc.

In other instances, smooth muscle contracting substances in lung exudates were measured by superfusing rat fundus strips according to the procedure of Vane [Br. J. Pharm. and Chemother. 12, 344 (1957)]. Recommended inhibitory drugs were employed in attempts to discriminate among the various possible smooth muscle contracting substances.

Succinoxidase activity. Lung tissue from ozone-exposed and air-control 200 g rats was treated according to the procedure of Mustafa, M.G., et al. [J. Lab. Clin. Med. 82: 357-365 (1973)]. Briefly, the tissue is homogenized in 10 volumes of dilute Tris buffer (pH 7.5) containing sucrose, mannitol and EDTA, and the homogenate is strained through cheese cloth. One ml of the homogenate is added to 0.5 ml of the modified Tyrode's solution containing succinate, ATP and hexokinase in a Warburg vessel; 6M KOH and a paper wick are added to the center well. Oxygen uptake is measured by the use of a Gilson Differential Respirometer.

Histamine forming capacity. This parameter was measured according to the procedure of Kahlson, G. et al, [Lancet 1: 782-784 (1966)]. Briefly, this procedure is as follows. Lung tissue (0.75 g) is minced with a mechanical mincer and added to 1.25 ml of 0.1 M phosphate buffer (pH 7.4) containing 2% glucose. To this are added 50 µL of histidine solution which contains 40 µg L-histidine (ring-2-^{14}C) having 4.4 µCi per mg of base. This mixture is incubated with shaking at 37°C under nitrogen for 3.0 hours. Then to the incubation mixture are added 66.2 mg of histamine-2 HCl and sufficient 5 M $HClO_4$ to make the final concentration 0.4 M. Histamine is separated from the supernatant fluid by ion-exchange chromatography and converted to the p-iodobenzenesulfonyl (pipsyl) derivative which is recrystallized and its specific activity determined by liquid scintillation spectrophotometry.

Angiotensin converting enzyme activity. Lung tissue samples remaining from the preparation of lung exudates were frozen and thawed twice and then homogenized in 0.05M phosphate buffer, pH 7.0, 2.0 ml per gram of tissue.

The homogenates were strained through gauze, frozen, and thawed twice and centrifuged for two hours at 2,000 x g. The supernatant solutions were diluted with 40 volumes of buffer (0.5M NaCl, 0.05M sodium phosphate (pH8.0), 0.05 per cent Brij 35) in order to reduce the converting enzyme activities of the lung extracts to levels comparable to those in serum and to provide the NaCl required for the activity of the lung enzyme.

The [^{3}H]-hippuryl-glycine-glycine containing "angiotensin converting enzyme activity radioassay system" from Ventrex Labs, Inc. (217 Read Street, Portland, ME 04103) was used to measure the enzyme activities in the diluted lung extracts which were expressed as nanomoles of hippuric acid generated per minute per gram of lung.

The uptake of circulating serotonin by lung. After having been exposed to ozone for 4 hours, the rats were anesthetized with Nembutal and the abdominal aorta cannulated. A tourniquet was applied to the neck of the animal and immediately thereafter 0.1 ml of saline containing 10 μg ^{14}C-serotonin (3-5 μCi/μmole) was injected into the inferior vena cava. Blood flowing out of the aortic cannula was then collected for the next minute in a tube containing heparin.

These blood samples were hemolyzed by freezing and thawing and were each diluted to 8.0 ml with distilled water. After the total proteins in each sample were precipitated by the addition of 2.0 ml of 40% trichloroacetic acid, one ml of the supernatant solution was added to 15 ml of scintillation cocktail and the total dpm determined. A 0.1 ml aliquot of the serotonin solution used for injection was treated similarly.

EXPERIMENTAL RESULTS

Succinoxidase activity. This measurement was included in the schedule of experiments in order to provide a link to previous investigations reported in the literature and as a check on our procedure of ozone exposure. Results of representative experiments, in which rats were exposed to one ppm ozone for four or sixteen hours, are given in Table I. These results are consistent with those of Mustafa et al. and indicate that the 4 hours exposure produces a decrease, whereas the 16 hours exposure produces an increase in succinoxidase activity in the lung.

Histamine forming capacity. Since many of the effects produced by lung damage have been attributed to histamine, it was of particular interest to determine the effect of ozone exposure on histamine release from lung tissue (see later) and on the histamine forming capacity of this tissue. Typical results obtained are given in Table II. They suggest that the histamine forming capacity of lung tissue of rats is not affected by either the exposure to one ppm ozone for 4 or 8 hours or by repeated exposures of four hours to two ppm ozone.

Preparation of lung exudates and non-histamine contracting substances. As a consequence of our preparation and assay of lung exudates, control experiments were performed using unexposed rats to ascertain the effect of the preparative procedure on the content of non-histamine smooth muscle contracting substances. Specifically, the effect of varying the time the lung pieces were in the Tyrode's solution was ascertained. The results obtained (Table III) indicate that while the concentration of non-histamine, smooth muscle contracting substance in the exudate may increase with the time allowed for the lung tissue to remain in the Tyrode's solution, the concentration has not significantly increased by the end of ten minutes.

In our routine procedure for the preparation of lung exudates, the pieces of lung stand in the Tyrode's solution for no longer than five minutes. It is apparent from the results obtained that the procedure employed would not contribute to the non-histamine smooth muscle contracting substances present in the exudates.

Effect of acute ozone exposure. A series of experiments were performed using the methods indicated above to ascertain the effects of exposing rats to ozone for 4.0 hours. The results obtained are in Table IV.

It is apparent that considerable variability in response to ozone exposure was encountered; the response of humans to ozone exposure also has been observed to vary considerably [Hackney, J.D. et al, Arch. Environ. Health 30: 378-384, 385-390 (1975)].

Table I. Succinoxidase Activity of Lung Tissue

Rats	Body Weight Range (g)	Ozone* Exposure Time (hrs.)	Oxygen Consumption (μL/vessel/10 min.)
Control	183-208	0	19.3 ± 0.48 (6)**
Exposed	172-208	4	15.5 ± 0.38 (6)†
Control	234-267	0	16.1 ± 0.37 (6)
Exposed	229-266	16††	19.1 ± 1.15 (6)†

* Ozone concentration equals 1.0 ppm.

** Mean ± S.E. Number of animals is in parentheses.

† $P < 0.02$; Student's "t" test.

†† Four hours per day for 4 consecutive days; animals sacrificed immediately after last exposure.

Table II. Histamine Forming Capacity of Lung Tissue

Rats	Body Weight Range (g)	Ozone Concentration (ppm)	Ozone Exposure Time (hrs.)	Specific Activity Fipsylhistamine (dpm/mg)
Control	195-218	0	0	4.28 ± 0.297 (5)*
Exposed	192-214	1.0	4.0	4.44 ± 0.297
Control	184-197	0	0	3.2 ± 0.41 (6)
Exposed	194-206	1.0	8.0	2.8 ± 0.68 (5)
Control	210-232	0	0	10.7 ± 1.12 (4)
Exposed	198-238	2.0	16.0**	9.3 ± 1.40 (2)

* Mean ± S.E. Number of animals is in parentheses. Differences from controls not significant; Student's "t" test.

** Four hours per day for 4 consecutive days; animals sacrificed immediately after last exposure.

Table III. Exudate Preparation Times

Group	Body Weight (g)	Lung Weight (g)	Time Lung in Tyrode's (min.)	Non-histamine Contracting Substance* (µg/g lung)
A	178 ± 4.2 (9)**	1.14 ± 0.034 (9)	one	3.50 ± 0.30 (9)
B	184 ± 2.6 (8)	1.11 ± 0.046 (8)	five	3.50 ± 0.53 (8)
C	181 ± 4.0 (7)	1.06 ± 0.048 (7)	ten	4.38 ± 0.27 (7)

* Determined by bioassay using guinea pig ileum treated with antihistamine and stropine. Units are µg histamine equivalents per gram lung.

** Mean ± S.E. Number of animals is in parentheses. No significant differences by ANOVA.

Table IV. Effects in Rats of Acute Ozone Exposure

Ozone Concentration (ppm)	Exposure Time (hrs.)	Body Weight (g)	Lung Water (per cent)	Lung Exudate Histamine (µg/g)	Lung Exudate Non-histamine* (µg/g)
0.0	0.0	245±9 (17)**	79.2±0.3 (16)	0.97±0.19 (17)	0.67±0.13 (17)
0.5	4.0	241±12 (6)	78.0±0.8 (6)	1.14±0.24 (6)	1.10±0.17 (6)†
1.0	4.0	251±7 (8)	80.1±0.5 (8)	0.91±0.21 (8)	2.22±0.57 (7)†
4.0	4.0	255±13 (7)	83.5±0.4 (6)†	1.18±0.24 (7)	0.41±0.11 (5)

* Non-histamine activity is the contraction observed in the presence of atropine and antihistamine (Pyrilamine, Merck); units are µg histamine or histamine equivalents per gram of lung.

** Mean ± S.E. The number of animals is in parentheses.

† Dunnett's test, $P < 0.01$. Percentages were converted to arcsine.

It is seen that 0.5 ppm ozone is effective in causing the release of non-histamine contracting substances into the exudates. The release of these substances is greater at an ozone concentration of 1.0 ppm, but, surprisingly, the control value was obtained when the ozone concentration was 4.0 ppm. This result possibly suggests that the non-histamine smooth muscle contracting substances released from lungs are sensitive to oxidation.

It is apparent that significant edema formation only occurred in these animals when the ozone concentration was 4.0 ppm. We also observed that histamine was not released by any ozone concentration equal to or less than 4.0 ppm. This result is consistent with the measurements of the histamine forming capacity presented above.

Thus, our results strongly indicate that the exposure of rats to ozone in concentrations of 0.5-1.0 ppm causes a release of non-histamine smooth muscle contracting substances. Since the assay procedure employed responds equally well to slow reacting substance of anaphylaxis (SRS-A) and to prostaglandins, the result is ambiguous. Because the release of these substances is not observed at an ozone concentration of 4.0 ppm, it was thought to be likely that the released substances are prostaglandins and that they are probably destroyed by the higher ozone concentration. Other explanations are also possible.

However, the results of exploratory experiments suggested that the released substances were indeed prostaglandins. Three rats weighing about 250g were given intraperitoneally 1.0 mg of indomethacin in 0.5 ml of 60% ethanol and one hour later were exposed to ozone at 1.0 ppm for 4.0 hours; three control rats received 0.5 ml of the ethanol at the same time as the experimental animals. The non-histamine contents of the lung exudates were: controls, 2.15±0.284 μg/g; experimental, 0.34±0.173 μg/g ($P<0.01$). Since indomethacin is a known inhibitor of prostaglandin synthetase (cyclooxygenase), this result strongly supports the possibility that at least some of the non-histamine smooth muscle contracting substances released into lung exudates during ozone exposure is prostaglandin.

Additional evidence as to the possible identity of the non-histamine smooth muscle contracting substances in lung exudates of rats exposed to ozone was provided by Dr. H. H. Tai, Texas College of Osteopathic Medicine, Denton, Texas. He analyzed pooled exudates from control rats and rats exposed to 1.0 ppm ozone for 4.0 hours by his radioimmunoassay procedure for thromboxane B_2 (TXB_2), the degradation product of the potent smooth muscle contracting substance thromboxane A_2 (TXA_2). It is a close relative of the prostaglandins and is derived, as are the prostaglandins, from the cyclooxygenase catalyzed reaction. He found the concentrations of TXB_2 to be 6 ng/ml and 10 ng/ml respectively in the control exudate and in the exudate from the ozone exposed rats. Our own bioassay of the two exudates gave non-histamine smooth muscle contracting activities equivalent to 1.1 and 2.0 μg histamine per ml of the pooled exudates from the control and ozone exposed rats, respectively.

Release of prostaglandin F_2 ($PGF_{2\alpha}$) and thromboxane A2. In view of the suggestive evidence which had accumulated, it was decided that a definitive

experiment should be performed. Therefore, male COBS Sprague-Dawley rats were procured (Charles River Labs) and exposed by the Health Effects Laboratory, Research Triangle Park, to ozone at a concentration of 1.0 ppm for 4.0 hours and afterwards the lung exudates were prepared. These exudates were analyzed by us using the bioassay and by Dr. Tai using his radioimmunoassay procedures for thromboxane B_2 ($TXB_2\alpha$), prostaglandin E_2 (PGE_2) and prostaglandin $F_2\alpha$ ($PGF_2\alpha$). The results are given in Table V.

It is seen that the concentrations of TXB_2 and $PGF_2\alpha$ were significantly greater in the exudates from the ozone exposed animals than in the control exudates; the concentrations of PGE_2 were the same in the two kinds of exudates. Since $PGF_2\alpha$ and TXA_2, the vasoactive substance measured as TXB_2, are both smooth muscle contracting substances in lung and since the results of the radioimmunoassays parallel the results of the bioassay, it was concluded that the non-histamine smooth muscle contracting substances released from the lungs of rats exposed to ozone must be comprised of $PGF_2\alpha$ and TXA_2, at least partially.

It is also noteworthy that exposure of these particular rats, i.e. COBS, to 1.0 ppm ozone for 4.0 hours produced significant lung edema as reflected by the increased lung weights in relation to body weights. Our previous experience (Table IV) indicated that exposure of rats to this concentration did not produce edema of the lungs.

Angiotensin converting enzyme activity (CEA). The samples of lung tissue remaining from the preparation of the lung exudates employed in Table V were used to assay the effect of ozone exposure on the converting enzyme activity (CEA) in lung. The method employed is described in the "Experimental Methods" section of this report.

Since in this procedure the lung homogenates were prepared by using volumes of buffers that were based on the fresh weights of the lung tissue, and since the lung weights of the exposed rats were larger than those of the controls, it was necessary to correct the initial results for this difference in weight which was undoubtedly due to edema. Since this edema fluid would act as a dilution factor, the initial results were corrected by multiplying them by the quotient of the exposed lung weights divided by the control lung weights. Results were (mean±SE): Air Controls - 3,569±116(9); Ozone exposed-(1.0 ppm - 4.0 hours.) 4,757±183(13) ($P<<.01$).

Please note that these results are nanomoles of hippuric acid generated per minute per gram of lung and that the pertinent information relative to the rats employed is in Table V.

Adaptation to ozone exposure in rats. The following question was posed: would the prior exposure of rats to a low dose of ozone affect the release of thromboxane A_2 and prostaglandin $F_2\alpha$ during a subsequent exposure to a high dose of ozone? To answer, we chose 0.5 ppm ozone for 4.0 hours as the first dose and 2.0 ppm ozone for 4.0 hours as the second dose.

Table V. Release of Prostaglandins by Ozone Exposure

Rats	Body Weight	Lung Weight	Lung Exudates: Non-histamine Activity*	Lung Exudates: TXB_2**	Lung Exudates: PGE_2**	Lung Exudates: $PGF_{2\alpha}$**
	(g)	(% b.w.)	(μg/g)	(ng/g)	(ng/g)	(ng/g)
Control	282 ± 20 (8)†	0.377 ± 0.0286 (8)	3.00 ± 0.639 (8)	15.3 ± 2.11 (6)	15.4 ± 2.63 (8)	8.4 ± 1.67 (8)
Exposed	249 ± 3 (20)	0.493 ±).0118 (18)††	6.82 ± 0.636 (10)††	25.0 ± 2.82 (8)††	14.7 ± 1.06 (8)	13.9 ± 1.17 (9)††

* See definition in footnote to Table IV.

** TXB_2=thromboxane B_2, PGE_2=prostaglandin E_2, $PGF_{2\alpha}$=prostaglandin $F_{2\alpha}$. Determined by Dr. H.H. Tai (Texas College of Osteopathic Medicine, Denton, Texas) using radioimmunoassay.

† Mean ± S.E. Number of animals is in parentheses.

†† Significantly different from control, $P \leq 0.02$, Student's "t" test.

In our previous experiments, exposure of rats to 0.5 ppm ozone for 4.0 hours was observed to be minimally effective in causing the release of the two mediators into the lung exudates while the exposure to 2.0 ppm ozone for 4.0 hours was definitely effective. The protocol was as follows: at 1:00pm of the first day, rats A and B were exposed to 0.5 ppm ozone for 4.0 hours and at 8:00 a.m. of the second day, rats B and C were exposed to 2.0 ppm ozone for 4.0 hours. At the end of this exposure, rats A, B, C and an unexposed rat (air control) were sacrificed and the lung exudates prepared. The total concentrations of the non-histamine smooth-muscle contracting substances in the exudates were determined by bioassay with the atropinized guinea pig ileum in the presence of the antihistamine, Pyrilamine, and expressed as µg equivalents of histamine. Concentrations of thromboxane B_2 (TXB_2), the stable degradation product of thromboxane A_2 (TXA_2), and of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) in the exudates were determined with radioimmunoassays by Dr. H. H. Tai, Texas College of Osteopathic Medicine, Denton, Tex. Table VI contains the results of these experiments.

It is seen that the exposure of rats to 0.5 ppm O_3 for 4.0 hours prevents the release of TXA_2 and $PGF_{2\alpha}$ into the lung exudates when the rats are exposed 15 hours later to 2.0 ppm O_3 for 4.0 hrs. This second dose of ozone is effective, however, in rats not previously exposed to ozone. This observation may explain the adaptation observed in human subjects upon exposure to 0.4-0.5 ppm ozone for three hours each day on five consecutive days, [Farrel, B.P. et al. Am. Rev. Resp. Dis. 119: 725-730 (1979)].

Of further interest is the observation that the higher ozone dose caused an increase in the weight of the lungs of both groups of rats, presumably due to edema formation. These results suggest that the release of TXA_2 and $PGF_{2\alpha}$ and edema formation may be dissociable phenomena. In addition, since the low O_3 dose is marginally effective in causing the release of the mediators and ineffective in causing edema (see Table IV), it would seem that the adaptation to O_3, which others have observed with respect to edema formation, may require that the first O_3 dose be definitely effective in producing edema. If these two effects of ozone exposure are indeed separable, then how does the release of TXA_2 and $PGF_{2\alpha}$ protect the rats from presumably lethal edema formation as a result of exposure to ozone?

The answer to this question is not apparent, but the results obtained with inhibitors of prostaglandin and thromboxane synthetases suggest that those mediators may be life protecting.

Prostaglandin/thromboxane inhibitors and ozone toxicity. Whether or not the release of the mediators $PGF_{2\alpha}$ and TXA_2 upon ozone exposure is beneficial to the rat has been examined in the following manner. Inhibitors of cycloxygenase and of thromboxane synthetase, both established and putative, are administered to rats and then these animals are placed in an ozone atmosphere. If the oxone is more toxic to animals which have received the inhibitors than to the controls, the tentative conclusion is that the mediator(s) concerned whose formation has been inhibited is protective. Experimental and control animals are exposed simultaneously and the survival times recorded. The results of these experiments are in Table VII.

Table VI. Development of Adaptation to Ozone Exposure

Rat Group	Body Weight	Lung Weight	Ozone Concentration	Lung Exudates: Non-histamine Activity*	Lung Exudates: TBX_2**	Lung Exudates: $PGF_{2\alpha}$**
	(g)	(g)	(ppm)	(μg/g)	(ng/ml)	(ng/ml)
A	274 ± 9 (10)†	1.26 ± 0.04 (10)	0.5	2.43 ± 0.528 (9)	5.95 ± 0.520 (8)	9.07 ± 1.287 (9)
B	272 ± 8 (10)	1.60 ± 0.07 (10)	0.5; 2.0	2.96 ± 0.585 (9)	6.08 ± 0.911 (9)	8.31 ± 1.011 (9)
C	275 ± 9 (10)	1.70 ± 0.13 (10)	2.0	6.40 ± 1.387 (9)	9.18 ± 1.226 (8)	13.84 ± 1.836 (9)
D	258 ± 7 (10)	1.15 ± 0.03 (10)	0	2.98 ± 0.416 (8)	5.98 ± 1.038 (10)	8.04 ± 0.785 (10)
Difference††	NS	B > D C > D C > A		C > A,B,D	C > A,B,D	C > A,B,D

Treatment description:

Group A - exposed to ozone for 4.0 hours; sacrificed 19 hours post-exposure.

Group B - exposed to ozone as was Group A and, after 15 hours, were exposed to 2.0 ppm ozone for 4.0 hours; sacrificed immediately thereafter.

Group C - exposed to 2.0 ppm ozone for 4.0 hours; sacrificed immediately thereafter.

Group D - controls.

* See definition in footnote to Table IV.

** See explanation in footnote to Table V.

† Mean ± S.E. Number of animals is in parentheses.

†† ANOVA; Duncan's new multiple range test, $\alpha = 0.05$, protection level is 86%.

Table VII. Ozone Toxicity and Inhibition of Prostaglandin Synthesis

Inhibitor	Dose Schedule	Ozone Concentration (ppm)	Survival Time (min.)	Change
None		4.5	439 ± 11 (15)*	
Indomethacin**	2.0 mg IP; 20, 15 and 0.5 hours preexposure	4.5	304 ± 27 (16)	Decreased, $P < .01$†
Ozone	0.5 ppm for 4.0 hours; 15 hours preexposure (see Table VI.)	4.5	419 ± 27 (8)	Decreased, $P < .05$†
None		7.0	267 ± 5 (31)	
Phenylbutazone**	15 gm IP; 0.25 hours preexposure	7.0	304 ± 11 (6)	No change††
Atropine Sulfate	20 mg IP; 1.25 and 0.25 hours preexposure	7.0	237 ± 5 (3)	No change††
Imidazole	40 mg in oil SQ; 16 and 0.25 hours preexposure	7.0	296 ± 36 (6)	No change††
1-Octylimidazole	10 or 15 mg IP; 1.5 hours preexposure	7.0	309 ± 24 (17)	No change††
None		6.5	247 ± 15 (11)	
Clotrimazole#	10 mg IP; 0.5 hours preexposure	6.5	355 ± 22 (11)	Increased, $P < .01$##

* Mean ± S.E. Number of animals is in parentheses.

** Inhibitor of cyclooxygenase (prostaglancin synthetase).

† Dunnett's test.

†† Duncan's new multiple range test.

Inhibitor of thromboxane synthetase.

Student's "t" test.

Not shown in this table are the toxicity studies with the drugs employed. In each case, when the drug was administered to normal rats in the dose indicated in Table VII, the animal lived in excess of 48 hours.

The increased ozone toxicity observed after the administration of indomethacin, a widely recognized inhibitor of cyclooxygenase (prostaglandin synthetase) and, therefore, of the synthesis of all prostaglandins and thromboxanes, indicates that the release of prostaglandins, thromboxanes, and/or prostacyclins is beneficial to the ozone exposed animal. Phenylbutazone administration has no effect even though this drug is also a cyclooxygenase inhibitor.

All of the inhibitors of thromboxane synthetase which were tried (imidazole, 1-octylimidazole, clotrimazole and atropine) prolonged the survival times of the exposed rats. However, this result was only significant in the case of clotrimazole, a specific inhibitor of thromboxane A_2 synthetase [Ladd, N. and Lewis, G.P., Br. J. Pharmac. 69: 3-5 (1980)]. The effect of this synthetic antifungal agent in lessening the toxic effects of ozone may be the result of the diversion of endoperoxide metabolism towards $PGF_{2\alpha}$ formation as a consequence of its inhibition of TXA_2 synthesis [Nijkamp, F.P. et al. Eur. J. Pharmac. 44: 179-186 (1977)]. If this presumption is true, then the apparent decrease in ozone toxicity produced by clotrimazole administration is in harmony with the concept that observed $PGF_{2\alpha}$ release is beneficial to the ozone exposed animal. Of course, the release of some other prostaglandin, which we have not measured, may also participate in this protective phenomenon.

Of particular interest among these experimental results is the observation that exposure of the rats to 0.5 ppm ozone 15 hrs. before the toxicity test decreased the survival time of the animals as compared to the controls. Previous experiments (see Table VI) indicated that in such pretreated rats 2.0 ppm ozone would not provoke the release of TXA_2 and $PGF_{2\alpha}$; the higher concentrations of ozone used in these toxicity experiments apparently do not cause the release of these mediators.

The effect of ozone exposure on the uptake of circulating serotonin by lung. By means of the procedure previously described, the in vivo abilities of lungs of normal rats and of rats exposed to 0.5 or 1.0 ppm ozone for 4.0 hours, to remove serotonin from the circulation, were determined. The results obtained are shown in Table VIII.

In confirmation of the work of others, the lungs of the control rats removed essentially all of the serotonin during one passage of the blood through these organs. A similar result would be expected with norepinephrine. However, exposure of the rats to ozone markedly and significantly reduced the ability of the lungs to remove serotonin from the blood. This effect of ozone exposure was still marked when the concentration of ozone was 0.5 ppm.

At this juncture, one must point out that the above effect can have at least two explanations. It may be due to lung congestion so that serotonin has less contact with lung tissue or the efficiency of the uptake mechanism may be decreased. Either reason is of interest. Perhaps analogous experi-

Table VIII. Uptake of Labeled Serotonin by Lung

Rat	Ozone Concentration (ppm)	Exposure Time (hrs.)	Radioactivity in Blood Sample (dpm)	Per Cent Dose Not Absorbed	Change from Control
Control			41 ± 11 (7)*	0.64	
Exposed	1.0	4.0	1271 ± 365 (7)	19.12	Increased, $P < .01$**
Exposed	0.5	4.0	813 ± 207 (6)	12.82	Increased, $P < .05$**

* Mean ± S.E. Number of animals is in parentheses.

** Dunnett's test.

ments using a labeled compound or micro beads which are not taken up by the lung cells would allow a decision as to which mechanism is operative.

The release of slow reacting substance of anaphylaxis (SRS-A). Even though the results of the foregoing experiments indicate that the non-histamine smooth muscle contracting activity in lung exudates of ozone exposed rats may be attributed to thromboxanes and prostaglandins, other active substances, such as SRS-A may also be present. Resolution of this question was attempted indirectly by administering methylprednisolone to prevent SRS-A release and directly by attempting to detect the presence of this mediator in lung exudates by bioassay.

Table IX contains the results of the experiments in which methylprednisolone was administered. This drug did not prevent the release of non-histamine smooth muscle contracting substances into lung exudates due to ozone exposure.

Figure 1 shows the response of rat fundus strips to lung exudates from ozone exposed rats in the presence of antagonists to acetylcholine (Ach), serotonin (5-Ht) and histamine (Hist.) The net contraction produced by the exudate from the ozone exposed rats disappeared when such rats were pretreated with indomethacin. It may be concluded, therefore, that exposure of rats to ozone (1.0 ppm, 4.0 hours), does not cause the release of SRS-A from lung. Rather, the prostaglandins released would appear to account for all of the non-histamine, smooth muscle contracting activity.

Table IX. Failure of Methylprednisolone to Inhibit Release of Non-histamine Activity

Rat	Body Weight (g)	Lung Weight (% b.w.)	Ozone Concentration (ppm)	Exposure Time (hrs.)	Methyl-prednis-olone*	Non-histamine Activity** (µg/g)
Control 1 "A"	255 ± 7 (4)†	0.494 ± .052 (4)				1.03 ± 0.589 (4)
Control 2 "B"	269 ± 1 (2)	0.450 ± .017 (2)			+	2.18 ± 0.0 (2)
Exposed "C"	281 ± 8 (6)	0.462 ± .025 (6)	1.15	4.0	+	3.16 ± 0.078 (5)
Difference††	NS	NS				C > A; P=.01 B > A; P=.05 C > B; P=0.1

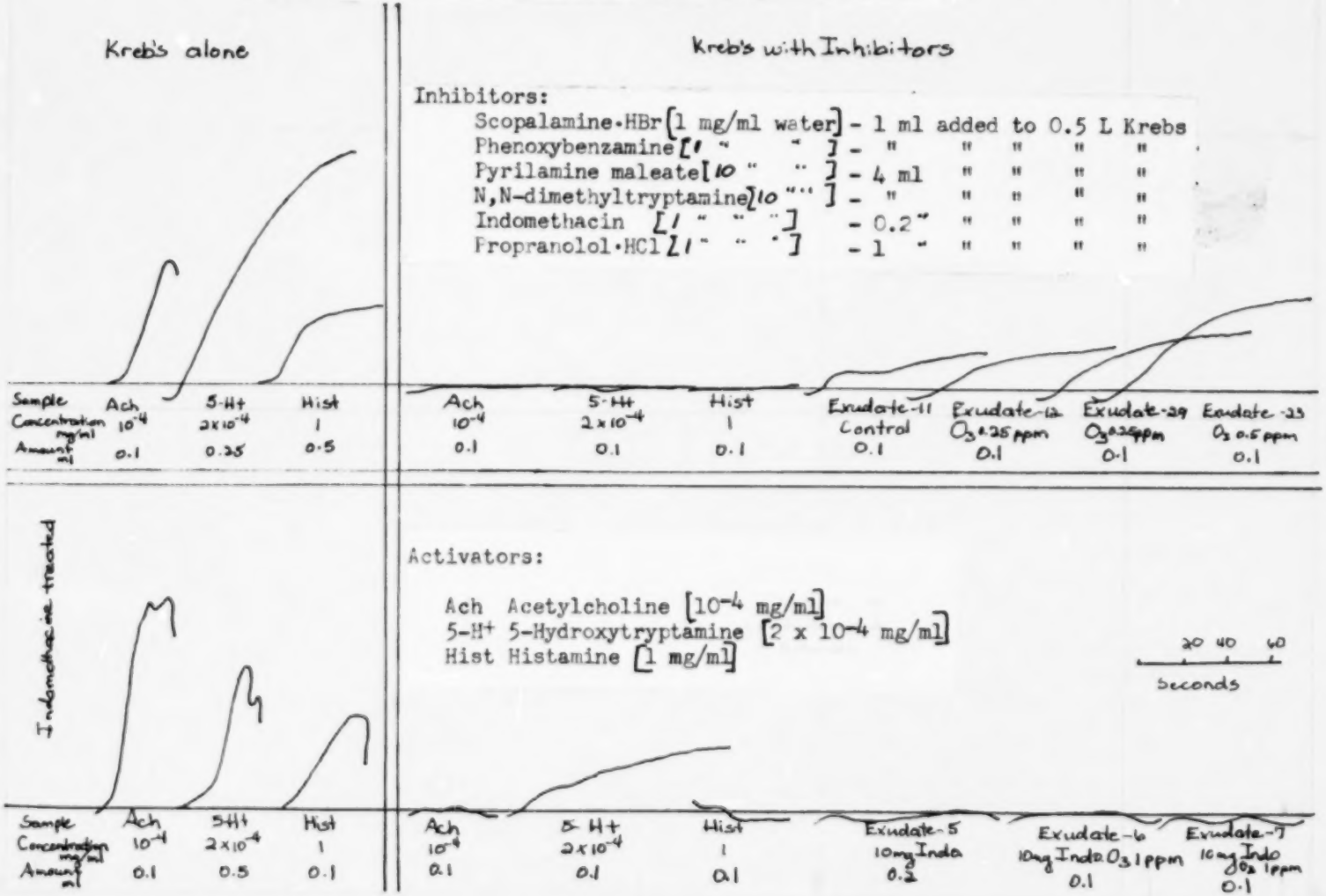
* 30.0 mg/kg body weight IP one hour pre-exposure.

** See definition in footnote to Table IV.

† Mean ± S.E. Number of animals is in parentheses.

†† ANOVA; Duncan's new meltiple range test.

Figure 1. Superfusion studies with rat fundus strips.


Krebs alone
Krebs with Inhibitors
Inhibitors:
Scopalamine·HBr [1 mg/ml water] - 1 ml added to 0.5 L Krebs
Phenoxybenzamine [1 " "] - " " " " "
Pyrilamine maleate [10 " "] - 4 ml " " " "
N,N-dimethyltryptamine [10 " "] - " " " " "
Indomethacin [1 " " "] - 0.2 " " " " "
Propranolol·HCl [1 " " "] - 1 " " " " "
Sample
Concentration mg/ml
Amount ml
Ach 10⁻⁴ 0.1
5-Ht 2x10⁻⁴ 0.25
Hist 1 0.5
Ach 10⁻⁴ 0.1
5-Ht 2x10⁻⁴ 0.1
Hist 1 0.1
Exudate-11 Control 0.1
Exudate-12 O3 0.25 ppm 0.1
Exudate-29 O3 0.25 ppm 0.1
Exudate-23 O3 0.5 ppm 0.1
Indomethacin treated
Activators:
Ach Acetylcholine [10⁻⁴ mg/ml]
5-Ht 5-Hydroxytryptamine [2 x 10⁻⁴ mg/ml]
Hist Histamine [1 mg/ml]
20 40 60
Seconds
Sample
Concentration mg/ml
Amount ml
Ach 10⁻⁴ 0.1
5-Ht 2x10⁻⁴ 0.5
Hist 1 0.1
Ach 10⁻⁴ 0.1
5-Ht 2x10⁻⁴ 0.1
Hist 1 0.1
Exudate-5 10mg Indo 0.2
Exudate-6 10mg Indo O3 1ppm 0.1
Exudate-7 10mg Indo O3 1ppm 0.1